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Probiotic *Escherichia coli* Nissle 1917 and Commensal *E. coli* K12 Differentially Affect the Inflammasome in Intestinal Epithelial Cells

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Key Words

Inflammasome · *Escherichia coli* Nissle 1917 · Crohn's disease

Abstract

Background: The probiotic bacterial strain *Escherichia coli* Nissle 1917 (EcN) is used for the treatment of ulcerative colitis (UC), diarrhea and constipation. Its beneficial effects in the treatment of UC have been demonstrated in several controlled clinical studies; however, the mechanism of action on the cellular level is still not completely clear. The intracellular pattern recognition receptor NLRP3 is expressed in intestinal epithelial cells (IEC), activates caspase-1 within the inflammasome complex and has been implicated to play a role in the etiology of inflammatory bowel diseases. **Methods:** Probiotic EcN and commensal *E. coli* K12 were applied to IEC in vitro. Inflammasome activation, interleukin (IL)-18 release and caspase-1 activation were determined by coimmunoprecipitation, Western blot and ELISA. Apoptosis was investigated by Western blot. **Results:** Incubation of Caco-2 cells with EcN resulted in lower inflammasome activation and subsequent secretion of mature IL-18 as compared to the commensal strain K12. Induction of apoptosis as determined by cleavage of caspase-3 and poly (ADP-ribose) polymerase were lower in EcN-stimulated cells. Autophagy was induced

by both bacterial strains, but to a higher extent by K12. **Conclusion:** These findings indicate that genetically very similar *E. coli* strains differ markedly in their ability to activate the inflammasome.

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Introduction

The inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC) are chronic, relapsing and remitting diseases that affect men and women equally, mainly starting in teenage years or young adulthood. IBD patients suffer from abdominal pain, diarrhea, rectal bleeding and weight loss [1]. It has become evident in recent years that both genetic and environmental factors play a role in the pathology of IBD. The general understanding of IBD is that they are caused by a defect of the intestinal barrier function, which can be partially assigned to hereditary influences.

The 'hygiene hypothesis' summarizes a number of approaches to explain increasing incidences of IBD in highly developed, industrialized countries. Similar to allergies and asthma, IBD shows an inverse relationship with the degree of sanitation. This indicates that factors influencing the composition of the commensal gut flora are likely to play a role in IBD [2].

The modulation of the gut flora by probiotic bacteria is efficient for the treatment of different forms of diarrhea, pouchitis and UC [3]. The current definition of probiotic bacteria was described by the World Health Organization in 2001 as probiotics being 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' [4].

Escherichia coli Nissle 1917 (EcN), unlike other common probiotics, is a Gram-negative bacterium. For the treatment of UC, beneficial effects of EcN were demonstrated in several controlled clinical trials, in which patients received either a standard therapy with 5-aminosalicylic acid (5-ASA) or EcN. The efficacy of EcN in maintaining remission was comparable to that of 5-ASA [5–7]. The mechanisms by which EcN exerts its positive effect are not clear. Studies with cell culture or animal models have identified several mechanisms, which might all contribute to the observed beneficial effect [8].

Comparing the effects of probiotic EcN and of the commensal strain *E. coli* K12 in intestinal epithelial cells (IEC), Kamada et al. [9] found that the presence of EcN suppressed TNF- α -induced interleukin (IL)-8 transcription and production. Wehkamp et al. [10] and Schlee et al. [11] showed that EcN triggered the expression and production of human β -defensin 2 (HBD-2) via NF- κ B signaling in Caco-2 cells. These findings are of particular interest with regard to IBD, since an altered defensin production has also been demonstrated for IBD patients [12, 13]. A promising approach to target this defensin deficiency is the construction of recombinant EcN which release HBD-2 [14].

Besides its function on inflammatory signaling, EcN also seems to positively influence the intestinal barrier function. The addition of EcN along with enteropathogenic *E. coli* strains prevented epithelial cell membrane disruption and induced an induction and redistribution of the tight junction protein zonula occludens 2 (ZO-2) in T84 cell cultures [15]. In mice, the induction of colitis by dextran sulfate sodium (DSS) and the parallel administration of EcN resulted in a less severe inflammation in both chronic and acute colitis models [16]. For the acute model, this effect was later shown to be associated with an upregulation of ZO-1 in IEC of mice that had been treated with EcN [17]. Another protective mechanism of EcN may arise from its ability to prevent adhesion and invasion of epithelial cells by other pathogenic bacterial strains [18, 19].

NLRP3 is an intracellular pattern recognition receptor which belongs to the family of NOD-like receptors (NLRs) of the innate immune system. Together with 'apoptosis-

associated speck-like protein containing a caspase recruitment domain' (ASC) and caspase-1, NLRP3 forms the inflammasome, an intracellular signaling platform that is activated upon a number of microbial and nonmicrobial stimuli [20]. Upon autocatalytic activation within the inflammasome complex, caspase-1 cleaves its substrates pro-IL-1 β and pro-IL-18 as well as pro-IL-33 into their mature isoforms, which are then released by the cell [21]. IL-18 exerts its function by binding to the IL-18 receptor (IL-18R) which is present on macrophages, natural killer cells, dendritic cells, and most T cells. Here, IL-18 induces the proliferation of its target cells and the transcription of IFN- γ and other proinflammatory cytokines [22]. The role of NLRP3 and IL-18 in IBD was demonstrated with experimentally induced colitis in mice. Nlrp3 knockout mice were shown to be more susceptible to DSS-induced colitis [23–25], accompanied by an impaired intestinal epithelial barrier function and an increased bacterial translocation [25]. In mice receiving DSS or TNBS for the induction of colitis, the concurrent treatment with IL-18-neutralizing antiserum or IL-18-binding protein ameliorated the inflammatory symptoms of colitis [26, 27]. Studies with transgenic mice revealed that overexpression of IL-18 leads to a more severe DSS-induced colitis with an increased infiltration of macrophages into the inflamed mucosa.

Elevated IL-18 serum levels were found in patients with UC, and this increase was correlated with the severity of inflammation [28]. Furthermore, IL-18 was more abundant in plasma and mucosa of CD patients as compared to control subjects [29–31]. Genome-wide association studies have identified NLRP3 as susceptibility gene for CD [32, 33].

The observed upregulation of proinflammatory IL-18 in IBD, and the fact that the inflammasome complex together with NLRP3 and caspase-1 is an activator of IL-18 led to the hypothesis that the beneficial effects of EcN might be mediated via this signaling pathway.

Methods

Bacterial Cultures

Two Gram-negative *E. coli* strains were used in this study. The probiotic strain EcN was cultivated from Mutaflor® capsules (Ardeypharm, Herdecke, Germany), and commensal *E. coli* strain K-12 (clone MG1655) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Brunswick). In their exponential growth phase in Luria Broth (LB) medium, bacteria were frozen in glycerol stocks. The number of colony-forming units (CFU) in stocks was determined by plating serial dilutions on LB agar plates.

Incubations and Stimulations

The IEC line Caco-2/BBE was cultured in DMEM high-glucose (Life Technologies, Grand Island, N.Y., USA) containing 10% FCS and 1% penicillin and streptomycin at 37°C and 10% CO₂. Prior to bacterial stimulation, the cell number was determined, and cultivation medium was exchanged by medium without serum and antibiotics. Low-dose stimulation by bacteria used a multiplicity of infection (MOI) of 0.1, meaning that the ratio is one CFU of bacteria to 10 eukaryotic cells. Stimulations were performed for up to 24 h with an MOI ranging from 0.1 to 100 as indicated.

SDS-PAGE and Western Blot

After stimulation, cells were harvested with M-Per lysis buffer (Thermo Fisher Scientific, Rockford, Ill., USA) and additionally lysed by sonication. Cytosolic fractions were diluted to 15 µg of total protein in NuPAGE sample buffer containing 50 mM dithiothreitol (Sigma-Aldrich, St. Louis, Mo., USA), denatured by boiling at 95°C for 5 min, before loading onto Bis-Tris SDS-PAGE gels. Proteins were transferred to nitrocellulose membranes (Life Technologies) which were subsequently blocked with 3% milk in Tris-buffered saline solution containing Tween-20 (TBS-T) for 1 h, and incubated with the primary antibodies overnight at 4°C. Primary antibodies against caspase-1 and poly (ADP-ribose) polymerase (PARP) were obtained from Cell Signalling (Danvers, Mass., USA), antibodies against occludin were obtained from Life Technologies, anti-IL-18 from Santa Cruz (Santa Cruz, Calif., USA) and anti-NLRP3 (NALP3b-γ) from Alexis (Lausen, Switzerland). After washing three times with TBS-T, secondary antibodies coupled to horseradish peroxidase (Santa Cruz) were diluted in 3% milk in TBST and incubated for 1 h at room temperature. Chemiluminescence detection used ECL solution from GE Healthcare (Little Chalfont, UK), and densitometric analysis was performed with Optiquant software (Packard Instrument Co., Meriden Conn., USA).

Coimmunoprecipitation

For coimmunoprecipitation (Co-IP), 100 µg of cytosolic fractions of protein lysates were used. In order to minimize unspecific binding to protein G sepharose beads (GE Healthcare), lysates were pre-cleared by rotating for 1 h at 4°C on a fraction of protein sepharose G which were then discarded. Only the supernatant containing pre-cleared lysate was used for immunoprecipitation with 1 µg of caspase-1 antibody (Santa Cruz) overnight at 4°C. On the following day, fresh protein G beads were added to each sample and allowed to bind to antibody precipitates for 1 h. After this, beads were spun down, washed with PBS, resuspended in sample buffer containing dithiothreitol and boiled for 10 min at 95°C. Supernatants were subjected to commercially available gradient 4–12% SDS-PAGE gels from Invitrogen, and subsequent Western blotting was performed as described above.

Quantification of Cytokine Secretion

A commercially available ELISA to detect active forms of IL-18 in cell culture supernatants was used according to the manufacturer's instructions (MBL International, Woburn, Mass., USA).

Statistics

One-way ANOVA analysis followed by post-hoc Tukey's Multiple Comparison Test was used to evaluate the significance of the results. For analysis, GraphPad Prism® Software 5.0 was used, and *p* values of <0.05 were considered statistically significant.

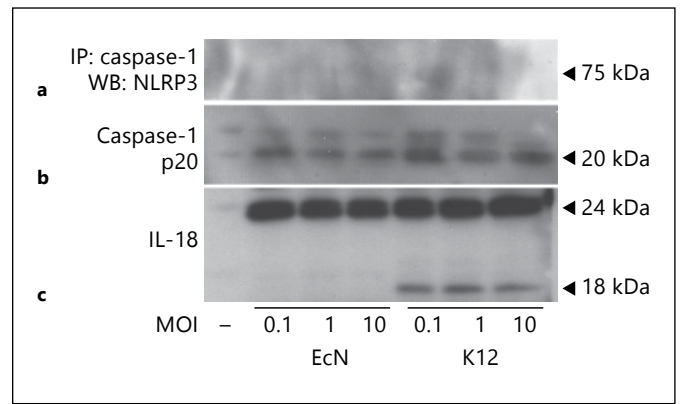


Fig. 1. Activation of inflammasome components upon bacterial stimulation. Cells were stimulated with indicated amounts of bacteria for 24 h. **a** Co-IP of caspase-1 and subsequent detection of NLRP3 in cells stimulated with K12 at an MOI of 0.1 revealed assembly of the two inflammasome components. **b** Caspase-1 activation was increased in cells stimulated with both strains of *E. coli* as compared to nontreated cells, but more pronounced after K12 stimulation. **c** IL-18 was detected as inactive proform at 24 kDa under all conditions except in untreated cells, whereas the cleaved subunit (18 kDa) was detected only in K12-stimulated cells. Western blot is representative of three independent experiments.

Results

Inflammasome Assembly Induced by EcN and *E. coli* K12

The assembly of NLRP3 with caspase-1 is a crucial step in inflammasome activation. By recruitment of caspase-1 to NLRP3 and ASC, caspase-1 is cleaved autocatalytically and can exert its action in cleaving its substrates, pro-IL-1β and pro-IL-18. The interaction of NLRP3 and caspase-1 was investigated by Co-IP. An increased assembly of caspase-1 and NLRP3 was found in cells treated with *E. coli* K12 already at an MOI of 0.1 (fig. 1a), reflected by the enhanced signal of NLRP3 as detected by Western Blot analysis. In line with this, the activation of caspase-1 was investigated by assessing the appearance of cleaved isoforms of 10 and 20 kDa (fig. 1b). Although an increase in the 20 kDa isoform was detected in both EcN- and K12-stimulated cells, it was clearly more pronounced in IEC stimulated with the commensal K12 strain.

Levels of Mature IL-18 Produced after Coincubation with EcN or *E. coli* K12

In the same experimental setup, we analyzed the turnover of one of the targets of caspase-1, namely pro-IL-18. Stimulation of CaCo-2 IEC with EcN did not induce the

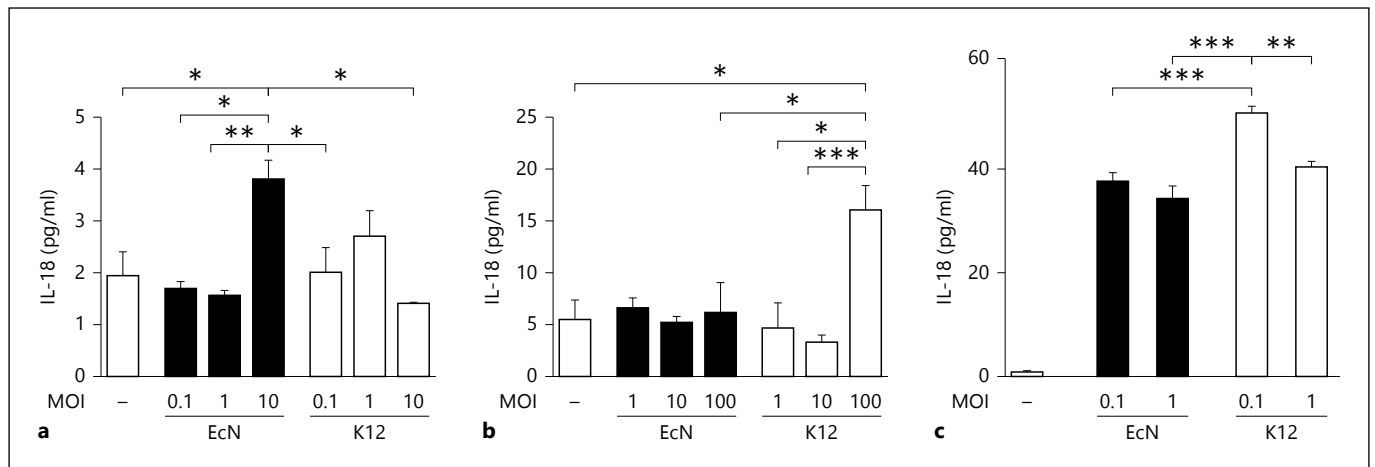


Fig. 2. Bacterial stimulation leads to IL-18 secretion. **a** IL-18 levels in cell culture supernatants of EcN- or K12-stimulated Caco-2 cells were assessed by ELISA after 6 h, which detected only small increases. **b** In a pulse-chase experiment, cells were stimulated for 1 h, washed and incubated for 24 h with antibiotics. **c** The release

of IL-18 was significantly increased after 24 h under all stimulation conditions as compared to nonstimulated cells. EcN caused lower secretion of IL-18 in both concentrations as compared to MOI 0.1 of K12-treated cells ($n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

production of detectable amounts of mature IL-18 in cytosolic lysates, while the non-cleaved (immature) pro-form was clearly present. In contrast, the commensal *E. coli* wild-type strain K12, applied in concentrations ranging from MOI 0.1 to 10 induced increased levels of mature (active) IL-18 (fig. 1c).

Once pro-IL-18 is cleaved by caspase-1, it is secreted by the cells, and can be detected in cell culture supernatants. A number of experiments were performed to better understand the kinetics of this activation and release. Stimulation with EcN or K12 for 6 h was not sufficient to trigger a robust activation and release of IL-18, as levels were close to the detection limit (fig. 2a). Since short incubation periods were not sufficient to reach solid IL-18 levels, a pulse-chase experiment was performed. A short, 1-hour incubation with indicated amounts of either EcN or K12 was followed by a washing step and further incubation of epithelial cells in bacteria-free, antibiotics-containing medium for 24 h, which was subsequently analyzed for the presence of active IL-18 by ELISA (fig. 2b). In cells stimulated with K12 at an MOI of 100, the short coincubation time resulted in the secretion of significantly higher amounts of IL-18 during the 24-hour period that followed K12 stimulation.

In the third experimental approach, cells were stimulated constantly for 24 h with low concentrations of both bacterial strains. To prevent bacterial overgrowth during the extended incubation period, only low concentrations of bacteria (MOI 0.1 and 1) were tested in this approach.

This was sufficient to trigger IL-18 release in substantial amounts (fig. 2c). Both strains induced a significant increase in IL-18 levels as compared to untreated control cells. Comparing the effects of EcN with that of *E. coli* K12, levels of IL-18 were found to be significantly higher in cells incubated with K12 at an MOI of 0.1. Thus, the incubation of IEC with probiotic EcN led to significantly lower levels of IL-18 as compared to stimulation with the commensal strain K12.

Induction of Apoptosis by EcN and *E. coli* K12

In the intestine of CD patients, increased apoptosis rates of IEC can be detected [34]. Therefore, we tested whether apoptotic events are altered by the stimulation of IEC with the two *E. coli* strains. Although the probiotic EcN strain induced a certain level of apoptosis in IEC, this effect was more pronounced in *E. coli* K12-treated cells.

Most apoptotic events lead to activation of caspases, among them the effector caspase-3. Activation of caspase-3 involves proteolytic cleavage and results in the appearance of the small subunit. In Caco-2 cells stimulated for 24 h with indicated concentrations of EcN or K12, this small subunit was detected by Western blot (fig. 3a). In contrast, no signal was observed in control cells, only the inactive pro-form was visible. The pro-form appeared more prominent in EcN-treated cells, suggesting less activation of caspase-3. Densitometric analysis and calculation of the ratio of total caspase-3 versus cleaved cas-

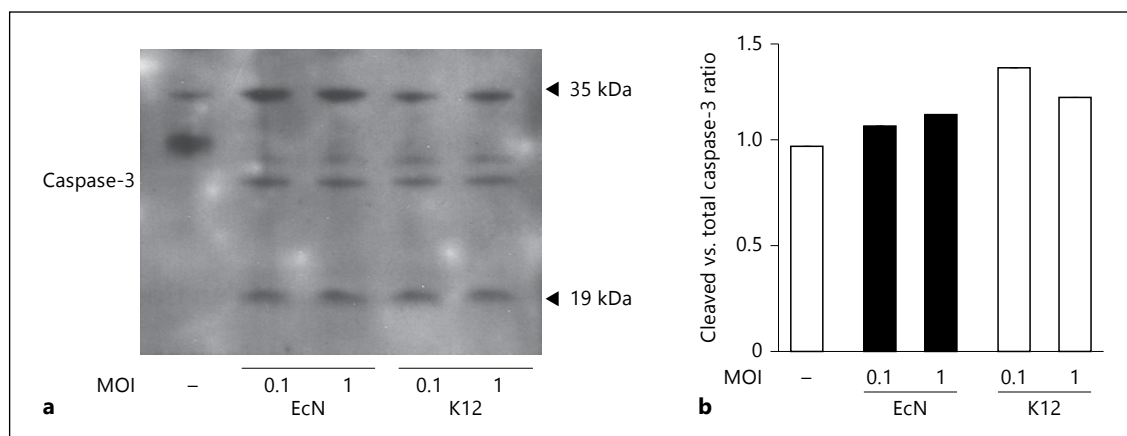
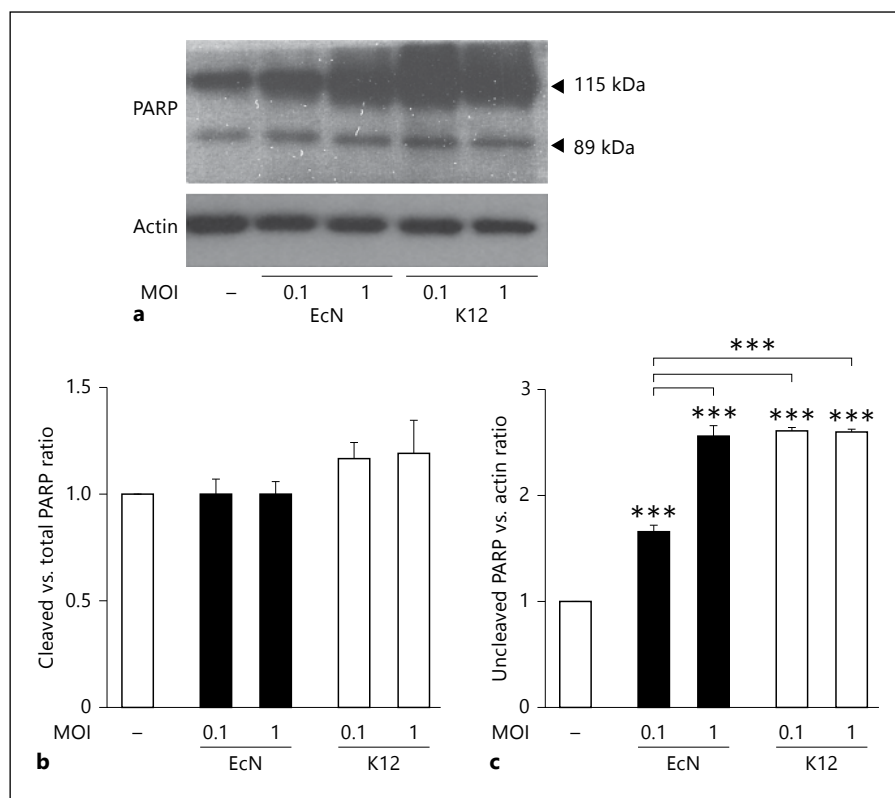


Fig. 3. Induction of apoptosis in IEC upon bacterial stimulation. **a** Caco-2 cells stimulated with bacterial strains showed elevated levels of activated caspase-3. **b** Densitometry points towards higher caspase-3 activation in K12-stimulated cells. Data are based on one experiment.

Fig. 4. Cleavage of PARP as a marker for apoptosis. **a** Cells stimulated with indicated amounts of EcN or K12 for 24 h displayed increased and dose-dependent cleavage of PARP. In K12-stimulated cells, more cleaved, thus inactivated, PARP was detected. **b** Densitometric analysis from three independent experiments revealed a trend towards higher rates of apoptosis only in K12-stimulated cells, although with no statistical significance. **c** Normalization of the densitometric analysis of the uncleaved PARP isoform to actin reveals that a general upregulation of PARP was present in EcN- and K12-stimulated cells as compared to untreated cells. Furthermore, a significant increase in PARP expression at higher EcN dose and both doses of K12 as compared to the cells stimulated with MOI of 0.1 was detected ($n = 3$, *** $p < 0.001$).

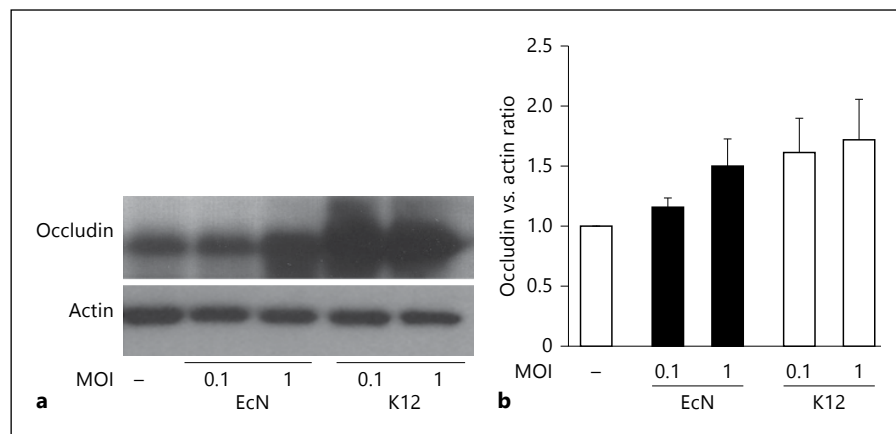


pase-3 revealed that cells stimulated with K12 showed more pronounced caspase-3 activation (fig. 3b).

In line with the finding that K12 triggers enhanced caspase-3 cleavage and thus apoptosis, we found that the target of caspase-3 PARP was cleaved more pronounced upon stimulation with the commensal strain of *E. coli*

(fig. 4a, b). Interestingly, not only the cleaved PARP product was present to a greater extent in K12-treated cells, but also the pro-form. The expression of uncleaved PARP was significantly higher in cells stimulated with K12 at a MOI of 0.1 as compared to EcN-treated and control cells.

Fig. 5. Occludin expression in response to *E. coli* stimulation. Protein expression of occludin appeared to be increased upon stimulation of Caco-2 cells with indicated concentrations of EcN or K12, although densitometric analysis and statistical analysis did not reach significance (n = 3).



A further prominent feature of CD is the finding that the intestinal barrier function is disrupted, allowing ions, water and bacteria to pass through the intestinal wall [35]. EcN has been reported to positively influence transepithelial resistance, causing upregulation of the tight junction protein ZO-1 in IEC [17]. To test whether another tight junction protein, occludin, is also regulated in IEC by the treatment with EcN or K12, the expression of occludin in Caco-2 cells after bacterial coinubation was examined by Western blot (fig. 5a). A slight increase in occludin expression was detected in all cells stimulated by K12 (MOI 0.1 and 1) and in those cells stimulated by EcN at an MOI of 1, although this increase was not statistically significant. The densitometric analysis and calculation of occludin versus actin expression demonstrated that occludin expression was induced in a dose-dependent manner, and to a higher extent in K12-stimulated cells (fig. 5b). As observed before with PARP, almost no changes were evident at the lower dose of EcN (MOI 0.1).

Contact of IEC with Bacteria Is Required for the Observed Effects

Interestingly, secreted factors of both bacterial strains had only mild effects on apoptosis and occludin expression. To test whether the previously observed effects in IEC could also be mediated without physical contact of bacteria to host cells, a transwell system was used. Caco-2 cells were grown in a 12-well plate, and bacteria were added to the apical compartment of a transwell membrane with an MOI of 1. Thus, bacteria were allowed to grow in close proximity of Caco-2 cells, but the transwell membrane kept them separated at all times. After 24 h, supernatants and cell lysates were harvested and analyzed by

ELISA and Western blot, respectively. PARP, a target of caspase-3, showed increased cleavage upon stimulation with any of the bacterial strains (fig. 6a, b). At the same time, the tight junction protein occludin appeared to be slightly upregulated by secreted factors of EcN and K12, but – as already stated above for occludin expression after direct stimulation with bacteria – densitometric and statistical analysis did not result in statistically significant changes of occludin expression (fig. 6a, c). Analysis of supernatants for the presence of IL-18 yielded no results, as IL-18 levels were below the detection limit (data not shown).

Discussion

The stimulation of IEC with low and high doses of probiotic EcN and commensal *E. coli* K12 resulted in a marked secretion of active IL-18 only in K12-stimulated cells. In line with this finding, NLRP3-inflammasome activation was observed mainly in cells stimulated with K12.

The comparison of probiotic EcN with commensal *E. coli* K12 reveals differences in NLRP3-inflammasome activation and the apoptosis pathway. Interestingly, assembly of NLRP3, activation of caspase-1 and secretion of IL-18 were all lower in EcN-treated cells as compared to cells treated with the commensal *E. coli* K12 strain. This is remarkable as there is a high genetic similarity between EcN and K12. Nevertheless, the inflammasome system or upstream signaling pathways obviously are able to discriminate between the two very similar bacterial strains.

Our findings support the concept that commensal *E. coli* cause more proinflammatory and proapoptotic sig-

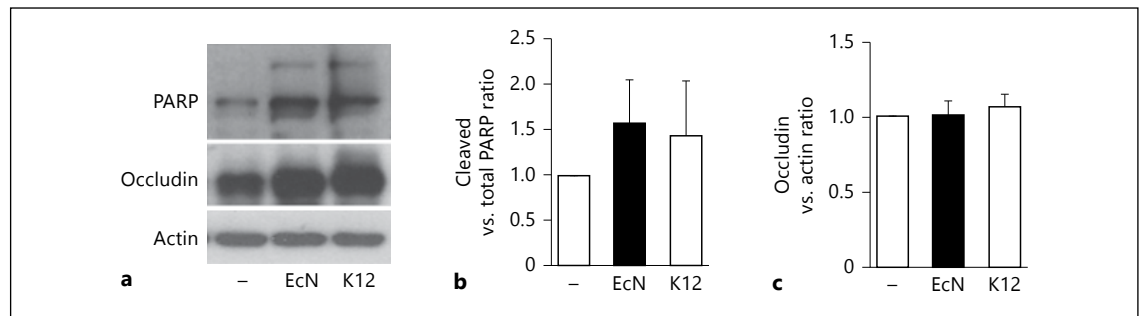


Fig. 6. Secreted factors of both bacterial strains have a mild effect on IEC. **a** Caco-2 cells were grown on a 12-well plate, and bacteria were added to the apical compartment of a transwell membrane with an MOI of 1. **b** PARP, a target of caspase-3, showed slightly increased cleavage upon stimulation of IEC with any of the bacte-

rial strains as determined by densitometric analysis. **c** At the same time, the tight junction protein occludin was not significantly up-regulated by secreted factors of EcN and K12. One Western blot detecting PARP, occludin and actin loading control is representative of three independent experiments.

naling in IEC compared to EcN, which has already been observed in professional immune cells before [36, 37].

Contrasting effects on cytokine stimulation in IEC have been described for a different probiotic bacterial strain of the species *Lactobacillus acidophilus* [38].

The cell culture model used in this study, of course, simplifies the situation that is normally present in the gut. Cells of the innate immune system, in addition, modulate IEC responses, and EcN has also pronounced effects on these cells [39].

How can the differential activation and assembly of the inflammasome by the two *E. coli* strains be explained? Certainly, an unselective binding of bacterial outer membrane-derived lipopolysaccharide to TLR4 cannot be the discriminator. TLR4 has been suggested to mediate a number of EcN effects in dendritic cells [40]. However, there are two arguments against an important role of TLR4 in our experimental setting. First, normal IEC express only low amounts of TLR4 (at least in our hands) [41]. Second, our data indicate that either a direct bacterial-epithelial cell contact or the presence of intact bacteria is required for the observed effects on inflammasome assembly. This would not be the case if TLR4 binding was sufficient to induce the investigated effects.

It is, however, possible that the reported upregulation of TLRs on the surface of IEC upon contact with commensal bacteria leads to an increase in substrates for inflammasome cleavage [42]. The TLR/MyD88 signaling pathway induces the proinflammatory transcription factors NF- κ B and AP-1, which, among others, induce the transcription of the genes for IL-1 β and IL-18, the substrates for caspase-1 cleavage. This can be regarded as the priming step for inflammasome activation, which ap-

pears to be necessary for efficient cleavage of pro-IL-1 β and pro-IL-18 by the NLRP3 inflammasome. The activation step of the NLRP3 inflammasome is still not fully understood, and several mechanisms have been proposed in the literature [43]. Apart from a priming step, the presence of extracellular ATP, subsequent activation of the P2X7 receptor and pannexin-1 pore formation leading to an efflux of K⁺ ions is a common hallmark preceding NLRP3 activation [20, 44]. The endocytosis of anorganic particles such as asbestos [45] and of crystals of uric acid [46] has been proposed to cause lysosomal rupture, which leads to the release of reactive oxygen species. Although commensal *E. coli* and EcN are noninvasive bacterial strains, the endocytosis of another *E. coli* strain into IEC was observed when metabolic stress was induced [47]. In this context, endocytosis and transcytosis may explain why otherwise noninvasive bacterial strains may be able to activate the intracellular pattern recognition receptor NLRP3. The elevated IL-18 levels, increased caspase-1 activation and NLRP3-inflammasome assembly observed in this study indicate an involvement of the NLRP3-inflammasome in response to these bacterial stimuli.

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Disclosure Statement

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